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Hepatocyte Growth Factor (HGF) Receptor Expression Is Inducible and Is Part of the Delayed-early Response to HGF*

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The c-*MET* proto-oncogene encodes the tyrosine kinase receptor for hepatocyte growth factor (HGF), also known as scatter factor, a potent mitogen and motogen for epithelial cells. The level of the HGF receptor expressed by epithelial cells varies in different growth conditions, being lower in growth arrested confluent monolayers and higher in growing sparse cells. The amount of HGF receptor mRNA increases from 3- to 5-fold after stimulation of confluent monolayers by serum and up to 10-fold after stimulation of protein kinase C by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). An increased level of the receptor mRNA was also observed after cell stimulation with nanomolar concentration of HGF itself. The effect was transient, dose, and time-dependent. Transcription of a reporter gene under control of the cloned 297 base pair c-*MET* promoter was also stimulated by serum, TPA, or HGF. The accumulation of specific mRNA is followed by appearance of the HGF receptor precursor protein, which is further processed to the receptor mature form. After HGF stimulation, HGF receptor expression follows c-*FOS* and c-*JUN* induction with a peak ~4 h. Pretreatment with the protein synthesis inhibitor puromycin strongly reduced the response to HGF, while cycloheximide alone increased the level of the receptor mRNA. These data show that c-*MET* behaves as a delayed early-response gene and suggest that the HGF response is autoamplified by inducing the specific receptor.

Hepatocyte growth factor (HGF),[1] also known as scatter factor, is a heterodimeric protein secreted by cells of mesodermal origin (Stoker *et al.*, 1987; Nakamura *et al.*, 1989; Weidner *et al.*, 1991). The factor induces a spectrum of biological activities in epithelial cells, including mitogenesis, stimulation of cell motility, and promotion of matrix invasion (Michalopoulos *et al.*, 1984; Nakamura *et al.*, 1984; Stoker *et al.*, 1987; Weidner *et al.*, 1990). HGF is also a morphogen *in vitro* (Stern *et al.*, 1990; Montesano *et al.*, 1991) and a potent angiogenic factor *in vitro* and *in vivo* (Bussolino *et al.*, 1992; Grant *et al.*, 1993). Both chains of HGF are encoded in a single gene (Miyazawa *et al.*, 1989; Nakamura *et al.*, 1989; Seki *et al.*, 1991), and native HGF is secreted as a biologically inactive single chain precursor (pro-HGF, Naldini *et al.*, 1992; Naka *et al.*, 1992). Activation takes place by proteolytic cleavage in the extracellular environment, and it is mediated by specific HGF convertases, including the urokinase-type plasminogen activator (uPA, Naldini *et al.*, 1992) and a novel serine protease homologous to the blood coagulation factor XII (Miyazawa *et al.*, 1993). Site-directed mutagenesis of a critical amino acid in the cleavage site generates a single chain biologically inactive HGF (Hartmann *et al.*, 1992; Lokker *et al.*, 1992), thus demonstrating that proteolytic cleavage is essential for acquisition of the active conformation.

HGF is the ligand for $p190^{MET}$, the receptor tyrosine kinase encoded by the c-*MET* proto-oncogene (Bottaro *et al.*, 1991; Naldini *et al.*, 1991a, 1991b). Cells transfected with the human c-*MET* cDNA express functional receptors and respond to HGF (Giordano *et al.*, 1993). The HGF receptor is a heterodimeric glycoprotein made of an extracellular α and a transmembrane β subunit (Giordano *et al.*, 1989a), both resulting from proteolytic cleavage of a common single chain precursor of 170 kDa (pr170: Giordano *et al.*, 1989b). The pleiotropic biological response induced by HGF indicates that activation of multiple intracellular signal transduction pathways are involved. Previous work has shown that the HGF receptor associates, upon autophosphorylation of specific tyrosines mapped at the C-terminal tail, with phosphatidylinositol 3-kinase, Ras-GAP, phospholipase C-γ, Src-related tyrosine kinases, and a tyrosine phosphatase (Graziani *et al.*, 1991; Bardelli *et al.*, 1992; Ponzetto *et al.*, 1993; Villa-Moruzzi *et al.*, 1993). Recently, we have also shown that HGF activates Ras by increasing the turnover between the GDP- and GTP-bound state through the stimulation of a guanine nucleotide exchange factor (Graziani *et al.*, 1993).

The finding that cell lines expressing a constitutively active HGF receptor tyrosine kinase are transformed, accentuates the oncogenic potential of the *MET* gene. This event occurs as a consequence of chromosomal rearrangements (Park *et al.*, 1986), gene amplification (Giordano *et al.*, 1989a; Ponzetto *et al.*, 1991), defective post-translational processing of the pr170 receptor precursor (Mondino *et al.*, 1991), or autocrine loops (Rong *et al.*, 1992). Overexpression of the *MET* oncogene has been observed in several human tumors of epithelial origin (Prat *et al.*, 1991a; Di Renzo *et al.*, 1991, 1992).

Little is known about the control of expression of the c-*MET* proto-oncogene. Under physiological conditions the HGF receptor is easily detectable in cells of epithelial origin, mainly localized in the proliferative compartments (Di Renzo *et al.*, 1991; Prat *et al.*, 1991a), in endothelial cells (Bussolino *et al.*, 1992),

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[1] The abbreviations used are: HGF, hepatocyte growth factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PIPES, 1,4-piperazineethansulfonic acid; SSC, saline sodium citrate; uPA, urokinase-type plasminogen activator; kb, kilobases; bp, base pairs.

in melanocytes (Natali *et al.*, 1993), and in microglial cells (Di Renzo *et al.*, 1993). The *MET* promoter has been cloned only recently (Gambarotta *et al.*, 1994).

In this work we show that expression of the HGF receptor is inducible at transcriptional level. The receptor expression varies according to the growing conditions of the cells and following stimulation with serum or phorbol esters. Interestingly, HGF itself up-regulates the mRNA levels of its own receptor and induces expression of uPA, an enzyme playing a key role in the activation of pro-HGF.

MATERIALS AND METHODS

Cell Culture—A549 is an epithelial cell line derived from a human lung adenocarcinoma. Cells were grown in RPMI 1640 medium supplemented with 10% volume/volume fetal calf serum (Flow Laboratories, Inc. McLean, Va) in a 5% CO_2-water-saturated atmosphere. Unless otherwise specified, cultures were rendered quiescent by growth to confluence and incubated for 48 h with RPMI 1640 medium supplemented with 0.1% (v/v) fetal calf serum, before receiving the additions specified in each experiment or before undergoing transfection.

Additions—Human HGF was purified to homogeneity, as described by Weidner *et al.* (1990), from the conditioned medium of Neuro 2A transfected with an expression vector under the control of the β-actin promoter (Bussolino *et al.*, 1992). Purified natural murine HGF, kindly provided by Dr. A. Coffer, was also used in some experiments. The preparations were tested for biological activity and used at a 50 ng/ml concentration unless otherwise stated. Human recombinant epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) were purchased from Sigma. Growth factors were kept as 5 μg/ml stock solution in 0.1% bovine serum albumin (radioimmunoassay grade) in H_2O. Solutions of the protein synthesis inhibitors puromycin and cycloheximide (Sigma, 10 mg/ml in H_2O) were freshly prepared on the day of use. 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 1-oleyl-2-acetyl-*sn*-glycerol, and α-phorbol-12,13-didecanoate were purchased from Sigma and prepared as 100 μM stock solutions in Me_2SO.

Western Blot Analysis and Antibodies—Total cellular protein were extracted solubilizing the cells in boiling Laemmli buffer (Laemmli, 1970). Samples were sonicated in order to decrease viscosity and adjusted to a protein concentration of 100 μg/each. For immunonoprecipitation, cells were lysed with ice-cold buffer containing 10 mM PIPES, pH 6.8, 100 mM NaCl, 5 mM $MgCl_2$, 300 mM sucrose, 5 mM EGTA, 1% Triton™ (Pierce), and protease inhibitors (Sigma). Extracts were clarified at 12,000 × *g* for 15 min and immunoprecipitated with an excess of anti-Met antibodies. These were polyclonal rabbit antibodies raised against a synthetic peptide corresponding to 19 C-terminal amino acids (from Ser^{1372} to Ser^{1390}) of the *MET* sequence (Ponzetto *et al.*, 1991; EMBL DataBank reference no. X54559). The immunocomplexes were collected on protein A-Sepharose, washed, and eluted in Laemmli buffer. Total cellular or immunoprecipitated proteins were electrophoresed in a 8% SDS-polyacrylamide slab gel under reducing conditions. Separated proteins were transferred to nitrocellulose paper (Hybond-C™, Amersham) by a graphite dry device (Electroblotter ™, Millipore). Blots were probed with anti-*Met* antibodies, followed by ^{125}I-labeled *Staphylococcus aureus* protein A (Amersham). Autoradiograms were exposed with an intensifying screen for 2–3 days. The molecular mass of labeled proteins was estimated relatively to the electrophoretic mobility of cotransferred ^{14}C-methylated protein standards (Amersham).

Northern Blot Analysis and Gene Probes—After cell stimulation, total cellular RNA was prepared using the single-step method of extraction described by Chomczynski and Sacchi (1987). For Northern blot analysis, 20 μg of total RNA were separated by electrophoresis on 0.8% denaturing agarose gels, transferred to nylon membranes (Hybond-N™, Amersham) by capillary action, and fixed according to the manufacturer's instructions. The gene probes used for hybridization were as follows. Glyceraldehyde-3-phosphate dehydrogenase (GADH) was a human full-length cDNA kindly provided by Dr. M. Pierotti. The c-*MET* probe was a full-length cDNA cloned from a GTL-16 (human gastric tumor cell line) library (Giordano *et al.*, 1993). The c-*FOS* (1 kb) and c-*JUN* (1.9 kb) probes were human cDNA fragments, kindly supplied by Dr. C. Santoro. Urokinase-type plasminogen activator probe was a human cDNA fragment (1.5 kb) generously provided by Dr. F. Blasi. Probes were labeled using the Megaprime™ DNA-labeling system with [α-^{32}P]dCTP, 3000 Ci/mmol (Amersham). Hybridization was carried out at 42 °C for 24 h in the presence of 50% formamide. Nylon membranes were washed under highly stringent conditions (0.1 × SSC, 0.1% SDS at 65 °C for 20 min) and underwent autoradiography using intensifying screens for 2 days. In some cases filters were stripped and reprobed in multiple cycles, according to the manufacturer's instructions.

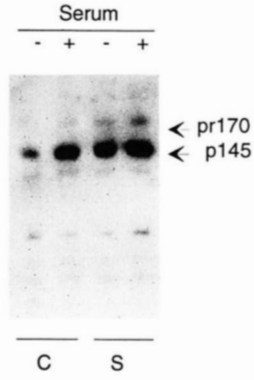

FIG. 1. **The level of HGF receptor expressed by epithelial cells varies in different growth conditions.** Confluent monolayers (*C*) or sparse (*S*) A549 cells were starved for 48 h in the presence of 0.1% fetal calf serum. 10% fresh serum (+) or medium alone (–) were then added for 24 h; after lysis, in the presence of β-mercaptoethanol, cells were analyzed in Western blot probed with antibodies against the C-terminal tail of HGF receptor. *Arrows* indicate the mature HGF receptor β chain (*p145*) and the uncleaved αβ precursor (*pr170*).

Transient Transfection and Luciferase Assay—A *Sma*I-*Sma*I DNA fragment encompassing the 297-bp 5′-flanking the transcription start site of the cloned c-*MET* gene (Gambarotta *et al.*, 1994) was recombined upstream to the luciferase reporter gene in the pGL2-Basic™ expression vector. The pGL2 vector carrying an SV40 promoter-luciferase construct was purchased from Promega. The expression vectors were complexed with Lipofectin™ (Life Technologies, Inc.) following the manufacturer's instructions and transfected in A549 cells during a 12-h incubation in Opti-MEM™ (Life Technologies, Inc.) serum-free medium. After transfection cells were stimulated in the presence of the specified additions for 36 h. Cells were solubilized and extracts were analyzed for luciferase activity using the Luciferase Assay System™ (Promega) after equalization of the protein contents.

RESULTS

The Level of HGF Receptor Expressed by Epithelial Cells Varies in Different Growth Conditions—Tissue culture dishes of A549 epithelial cells, containing either confluent monolayers or sparse growing cells, were solubilized and analyzed by Western blot with anti-*Met* antibodies (Fig. 1). The amount of HGF receptor expressed by quiescent cells packaged in confluent monolayers was low. Stimulation of monolayers by addition of fresh medium containing fetal calf serum (10%) was followed by a wave of thymidine incorporation (data not shown), and enhanced expression of HGF receptor. The level of receptor expressed by sparse cells was already high, comparable to the amount of receptor detectable in serum-stimulated monolayers, and did not further increase by addition of fresh serum. A slight increase in the amount of unprocessed receptor precursor (*pr170*) was however observed (Fig. 1).

HGF Receptor mRNA Accumulates in Response to Cell Stimulation by Serum or TPA—The effect of serum stimulation on the level of mRNA encoding the HGF receptor was measured in A549 cells by Northern blot analysis. As described, hybridization with a full-length cDNA probe detected a major c-*MET* transcript conventionally indicated as 9 kb (Park *et al.*, 1986; Di Renzo *et al.*, 1991). This encompasses the coding sequence for the complete HGF tyrosine kinase receptor. This transcript, already detectable in resting cells grown *in vitro*, increased up to 3–5-fold in serum-stimulated cells. The increase was transient, with a maximum ~4 h (Fig. 2*A*). The 9-kb c-*MET* mRNA was significantly increased (~10-fold) also in cells stimulated by the protein kinase C activator TPA. The effect was observed at concentrations within the nanomolar range and was long lasting. There was a notable increase after 4 h which continued up to 16 h (Fig. 2*B*). Similar effects were observed after stimu-

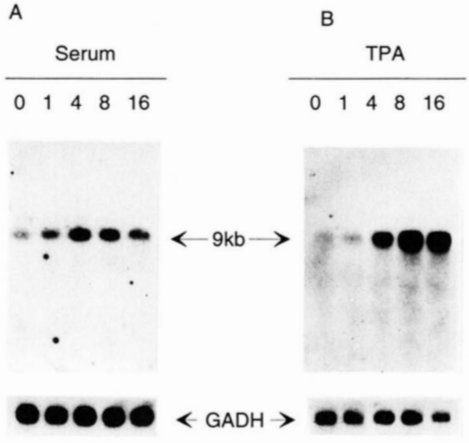

FIG. 2. **HGF receptor mRNA accumulates in response to cell stimulation by serum or TPA.** Serum-starved confluent monolayers were stimulated by addition of fresh medium containing 10% fetal calf serum (*panel A*) of 160 nM TPA (*panel B*). Total mRNA was extracted after the indicated times (1–16 h) and analyzed in Northern blot with a full-length c-*MET* cDNA probe. *Arrows* indicate the major transcript encoding the HGF receptor, conventionally designated as 9 kb. A probe for the housekeeper gene glyceraldehyde-3-phosphate dehydrogenase (*GADH*) was used as a control to evaluate the amount of mRNA transferred to filters. The Northern blot shown in *panel A* was exposed for 48 h, the blot shown in *panel B* for 24 h.

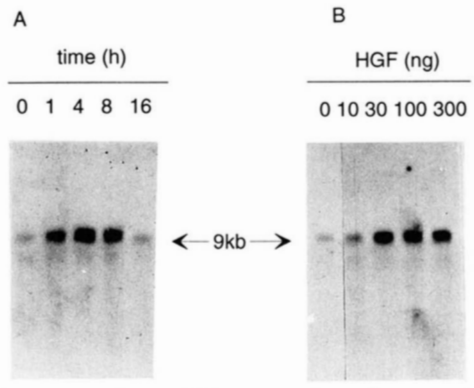

FIG. 3. **HGF receptor mRNA accumulates in response to cell stimulation with HGF.** *Panel A*, serum-starved confluent monolayers were stimulated by addition of a fixed dose of HGF (50 ng/ml) and analyzed in Northern blot after the indicated times. *Panel B*, monolayers were stimulated by increasing concentrations of HGF, as indicated, and total RNA was analyzed after 4 h. Both filters were hybridized with the c-*MET* cDNA probe. *Arrows* indicate the major transcript encoding the HGF receptor (9 kb). No significant differences in the RNA lane loading were observed when probing the filters with a cDNA for the housekeeper gene glyceraldehyde-3-phosphate dehydrogenase (not shown).

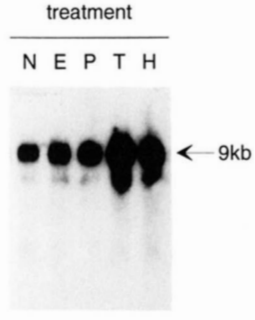

FIG. 4. **Levels of HGF-receptor mRNA in cells treated with different growth factors.** Serum-starved confluent monolayers were stimulated by addition of EGF (5 ng/ml, *E*), PDGF (5 ng/ml, *P*), TPA (160 nM, *T*), or HGF (50 ng/ml, *H*). After 4 h, the mRNA underwent a Northern analysis using the c-*MET* cDNA probe. *N*, control unstimulated cells. *Arrows* indicate the major transcript encoding the HGF receptor (9 kb). No significant differences in the RNA lane loading were observed when probing the filters with a cDNA for the housekeeper gene glyceraldehyde-3-phosphate dehydrogenase (not shown).

lation with another protein kinase C activator, the natural compound 1-oleyl-2-acetyl-*sn*-glycerol while no stimulation was observed with α-phorbol 12,13-didecanoate, a phorbol ester unable to activate protein kinase C (not shown).

HGF Receptor mRNA Accumulates in Response to Cell Stimulation by HGF Itself—The level of the HGF receptor 9-kb transcript increases also after treatment with recombinant HGF, as assessed by Northern blots hybridized with a full-length c-*MET* probe. Stimulation of serum-starved confluent monolayers induced accumulation of HGF receptor mRNA up to 5-fold. The increase was transient, significant after 1 h, and with a peak stimulation around 4 h. After 16 h the amount of specific mRNA returned to the basal level (Fig. 3*A*). The HGF-induced increase in HGF receptor mRNA was dose dependent and maximum stimulation was reached within the nanomolar range (Fig. 3*B*). The differential induction by TPA and HGF with regard to time course may be explained by the different kinetics of interaction with their targets. The ligand-induced activation of the HGF receptor and the downstream intracellular signal cascade is rapid and reversible (for a review see Comoglio, 1993). The interaction between TPA and protein kinase C is slower, but the activation of the downstream response is persistent (Shoyab and Todaro, 1980; Kikkawa and Nishizuka, 1986).

A549 cells are of epithelial origin and express receptors for EGF: after stimulation with EGF no significant increase of the HGF receptor mRNA was observed within the first 4 h. PDGF had no effect (Fig. 4).

The HGF Receptor Gene (c-MET) Promoter Is Stimulated by Serum, TPA, or HGF—To asses whether HGF receptor expression is regulated at transcriptional level, we transfected A549 cells with a construct carrying the luciferase reporter gene under the control of the first 297 bp upstream to the transcription start site of the HGF receptor gene. This sequence has been shown to contain a number of consensus elements for known transcriptional factors and to be a functional promoter (Gambarotta *et al.*, 1994). Cells were starved for 48 h in low serum (0.1%) and transfected in serum-free medium. When further kept in low serum, transcriptional activity was barely detectable. When cells were stimulated with 10% serum after transfection, a remarkable increase in the activity was measured. A lower, but significant stimulation of transcriptional activity was also observed when they were incubated in the presence of TPA or HGF (Fig. 5).

Accumulation of Specific HGF Receptor mRNA Is Followed by Translation and Processing of Mature HGF Receptors—Upon TPA or HGF treatment the accumulation of specific mRNA was followed by the appearance of the HGF receptor precursor and of the mature receptor. As previously shown (Giordano *et al.*, 1989b), the precursor is a single chain glycoprotein of 170 kDa (pr170): the molecule undergoes intracellular cleavage generating the mature heterodimeric form, composed of a 50-kDa α chain covalently linked to a 145-kDa β chain (p145). Stimulation of A549 cells by TPA causes a striking increase in the amount of pr170 and of p145. A significant increase of both proteins was also observed in cells treated with HGF (Fig. 6*A*). Time course experiments showed that, after TPA treatment, the mature HGF receptor accumulates after 4 h and reaches a plateau at 8–12 h. The maximum increase in the precursor level was reached earlier, due to the rapid processing of pr170 (Fig. 6*B*). A similar kinetics was observed in cells stimulated by HGF (data not shown). A smaller accumulation of HGF receptor protein was also observed upon stimulation with serum (not shown).

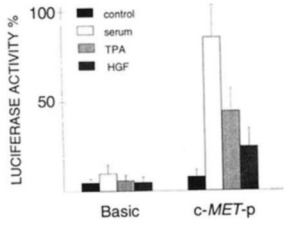

FIG. 5. **Transcriptional activity of the isolated c-*MET* promoter.** Serum-starved confluent monolayers were transfected with expression vectors containing the luciferase gene under control of the 297-bp c-*MET* functional promoter (*c-MET-p*), or luciferase without promoter (*Basic*). Transfected cells were incubated for 36 h in the presence of 0.1% serum (*control*), or 10% serum, 160 nM TPA or 50 ng/ml HGF. The histogram shows the average luciferase activity measured in three independent experiments. Values are percentages of the transcriptional activity of a transfected SV40 promoter, measured after 36 h in serum-free medium.

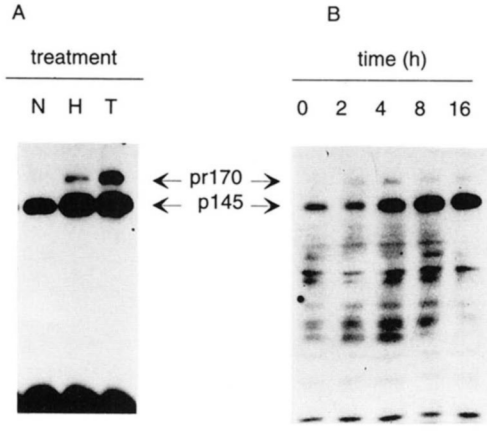

FIG. 6. **Accumulation of specific mRNA is followed by translation and processing of mature HGF receptors.** *Panel A*, confluent monolayers were incubated for 4 h with HGF (*H*) or TPA (*T*), then lysed, and immunoprecipitated with anti-Met antibodies. Immunocomplexes were reduced and further analyzed by Western blot probed with the same antibodies and radiolabeled protein A. *N*, untreated control cells. *Panel B*, cells were stimulated in the presence of TPA (160 nM) and lysed after the indicated time intervals (*h*). Total proteins were analyzed by Western blot probed with anti-Met antibodies. *Arrows* indicate the mature HGF receptor β chain (*p145*) and the uncleaved αβ precursor (*pr170*).

The HGF Receptor Gene (c-MET) Behaves as a Delayed-early Response Gene—The above experiments show that the expression of the HGF receptor is controlled by serum, phorbol esters, and HGF itself. These data suggest that c-*MET* behaves as a growth factor response gene of either the early or delayed type. Time course experiments were then performed comparing accumulation of c-*MET* mRNA with that of mRNA transcribed from immediate-early genes, such as c-*FOS* and c-*JUN*, or from a delayed-early gene such as the one encoding the uPA. Fig. 7 shows that, in response to HGF, the mRNA of c-*FOS* and c-*JUN* transiently increases with a peak at 30 min, the c-*MET* mRNA follows with a peak ~4 h, and the uPA mRNA raises slower reaching a plateau after 8 h.

Discrimination between immediate-early and delayed-early response genes is also achieved by measuring the mRNA response in the presence of protein synthesis inhibitors such as puromycin (Lanahan *et al.*, 1992; Lau and Nathans, 1991). In the presence of the drug, HGF-induced accumulation of HGF receptor mRNA was inhibited in a dose-dependent manner (Fig. 8*A*). Complete inhibition of HGF-induced mRNA accumulation was observed at concentrations of puromycin ≥20 μg/ml. The same doses of puromycin alone only slightly inhibited the basal level of HGF receptor mRNA. Twenty μg/ml of the drug fully inhibited cellular protein synthesis, as assessed by

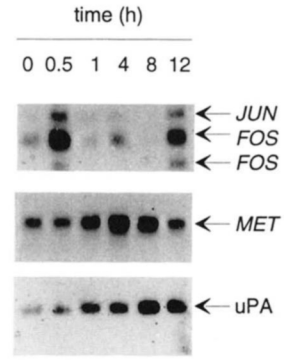

FIG. 7. **Elevation of mRNAs in response to HGF.** Cells were stimulated with HGF, lysed after the indicated times (*h*), and analyzed in Northern blot. The same filter was hybridized repeatedly with cDNA probes for the genes c-*JUN*, c-*FOS*, c-*MET*, and for urokinase (*uPA*). The c-*JUN* probe hybridized with one transcript, of about 2.7 kb; the c-*FOS* probe with two transcripts, estimated respectively about 2.4 and 1.9 kb; the c-MET probe with the 9 kb major transcript; the probe for uPA with a single transcript of about 2.5 kb. No significant differences in the RNA lane loading were observed when probing the filters with a cDNA for the housekeeper gene glyceraldehyde-3-phosphate dehydrogenase (not shown).

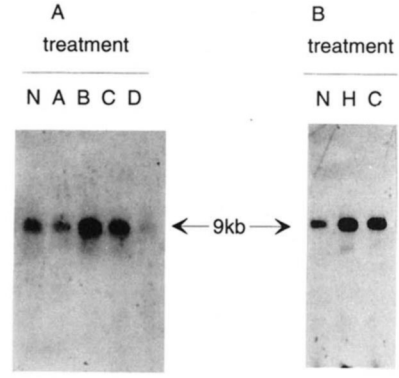

FIG. 8. **HGF receptor mRNA elevation in the presence of protein synthesis inhibitors.** Different plates of serum-starved confluent monolayers were incubated as follows. *Panel A*: *N*, control medium; *A*, puromycin alone (20 μg/ml); *B*, HGF alone (50 ng/ml); *C*, HGF (as above) and puromycin (10 μg/ml); *D*, HGF (as above) and puromycin 20 μg/ml. *Panel B*: *N*, control; *H*, HGF, as above; *C*, cycloheximide (10 μg/ml). After 4 h total RNA was analyzed by Northern blot and hybridized with the c-*MET* cDNA probe. *Arrows* indicate the major transcript encoding the HGF receptor (9 kb). No significant differences in the RNA lane loading were observed when probing the filters with a cDNA for the housekeeper gene glyceraldehyde-3-phosphate dehydrogenase (not shown).

[^{35}S]methionine incorporation but did not suppress the induction of the immediate-early gene c-*FOS* by HGF (data not shown). In contrast, the accumulation of c-*MET* mRNA in A549 cells was stimulated by cycloheximide (Fig. 8*B*). It has already been demonstrated that this protein synthesis inhibitor elicits a number of biochemical effects resulting in the induction of TPA response genes, such as the urokinase receptor (Lund *et al.*, 1991) or immediate-early proto-oncogenes (Edwards and Mahadevan, 1992).

DISCUSSION

Little is known about the regulation of expression of growth factor receptors of the tyrosine kinase family. In this report we show that, *in vitro*, the level of HGF receptor is lower in growth-arrested confluent epithelial cells and higher in sparse growing cells. Recruitment in the growing state by stimulation with fresh serum results in a significant increase of both mRNA and receptor protein. The insulin receptor behaves contrarily: the receptor mRNA level raises in growth-arrested cells (Levy and Hug, 1992). Although the two receptors are distantly related

(Hanks *et al.*, 1988), this observation is not surprising since the biological responses elicited by HGF or insulin are quite different (Gherardi and Stoker, 1991; Rosen, 1987). HGF is a powerful mitogen and motogen, therefore, increased expression of its receptor in growing cells may have a role in the progression between the phases in the cell cycle.

The data reported demonstrate that protein kinase C activation by TPA strongly induces expression of HGF receptor. Previous work from this laboratory has shown that, following TPA treatment, an immediate serine phosphorylation and concurrent tyrosine dephosphorylation of the receptor exposed at the cell surface occurs (Gandino *et al.*, 1990; 1991). Moreover, TPA induces rapid proteolytic cleavage of the receptor β chain, generating a truncated extracellular fragment lacking the cytoplasmic tyrosine kinase domain (Prat *et al.*, 1991b). Both mechanisms result in the inactivation of the signal transduction mediated by the HGF receptor. This negative modulation takes places within minutes after TPA treatment. The data now reported show that subsequently (hours) protein kinase C activation up-regulates receptor synthesis, inducing both mRNA accumulation and protein translation. A similar late effect of protein kinase C on EGF receptor has been described by Bjorge and Kudlow (1987), who have shown that receptor synthesis rate increases 5-fold in cells stimulated by phorbol esters.

Interestingly, in cells grown *in vitro*, HGF receptor expression is also induced by HGF itself. A similar behavior was again shown for the EGF receptor: EGF stimulation increases receptor mRNA levels by 3–5-fold and stimulates receptor synthesis within a few hours (Clark *et al.*, 1985; Kudlow *et al.*, 1986; Earp *et al.*, 1986; Bjorge and Kudlow, 1987). In the case of HGF receptor, induction by the cognate growth factor seems also to operate *in vivo*. In this respect, it has been found that partial hepatectomy, or CCl_4-induced hepatocellular necrosis, cause a dramatic rise in the level of circulating HGF (Lindroos *et al.*, 1991). Within a few hours, this rise in HGF is followed by increased expression of HGF receptor mRNA in the regenerating hepatic tissue, both in the case of hepatectomy[2] and of CCl_4 intoxication (Ito *et al.*, 1993). Whether HGF receptor is specifically induced by HGF only is unclear. Among the other growth factors tested on the A549 cells used in this study, PDGF was ineffective and EGF was able to elicit a modest increase of HGF receptor mRNA only after several hours (12–15 h; data not shown). The signal transduction pathway used by HGF to up-regulate the expression of its own receptor remains to be elucidated.

HGF receptor is encoded by the c-*MET* proto-oncogene. The recent cloning of the c-*MET* promoter (Gambarotta *et al.*, 1994) gives some insight on the mechanism(s) that are likely to operate in the increased expression of the receptor after serum, TPA, or HGF stimulation. The promoter, encompassing at least 297 base pairs upstream to the start site of transcription, contains a number of *cis*-acting elements including a consensus sequence for the transcriptional factor PEA3, at position −82, and two AP2 sites, at positions −84 and −166. The PEA3 motif is known to be responsive to TPA and to growth factors (Xin *et al.*, 1992). AP2 regulatory elements mediate the induction of transcriptional activity after stimulation by TPA (Imagawa *et al.*, 1987). The transcriptional activity of the isolated *MET* promoter, transfected in A549 cells, was low in resting cells and was stimulated by serum, by TPA, or by HGF. The data strongly suggest that these factors may operate at transcriptional level also on the endogenous promoter.

The c-*MET* proto-oncogene behaves as a growth response gene, as it is induced by serum, phorbol esters, and HGF. From the comparison of the HGF induced expression of c-*FOS*, c-*JUN*, uPA, and c-*MET* genes in time course experiments, it was concluded that the latter is to be included in the group of the genes expressed in the delayed-early response (Lanahan *et al.*, 1992; Lau and Nathans, 1991). This conclusion is supported by experiments of suppression of c-*MET* induction by puromycin, a well known protein synthesis inhibitor. In fact, protein synthesis is required for delayed-early genes activation (Lanahan *et al.*, 1992; Lau and Nathans, 1991). In contrast, cycloheximide alone was found to induce accumulation of HGF receptor mRNA. The same effect of cycloheximide has been reported in the case of mRNA accumulation for the uPA receptor gene and for c-*FOS* and c-*JUN*. This phenomenon has been correlated with the capacity of the drug to mimic the nuclear signaling responses elicited by EGF or TPA (Lund *et al.*, 1991; Edwards and Mahadevan, 1992).

The data reported in this paper show that HGF receptor is inducible and that its expression is increased in response to HGF itself. It is also worth noting that another HGF-induced gene is uPA, the urokinase-type plasminogen activator, known to be involved in the proteolytic activation of the HGF precursor (Naldini *et al.*, 1992). This induction occurs at mRNA (Pepper *et al.*, 1992) and protein level (data not shown). The inactive pro-HGF is widely distributed in tissues, mainly bound to the extracellular matrix (Naldini *et al.*, 1992). The amount of receptor expressed by neoplastic cells is abnormally high, even in the absence of amplification of the *MET* gene (Di Renzo *et al.*, 1991, 1992; Prat *et al.*, 1991a). We would like to propose that HGF responsiveness may be autoamplified by a paracrine mechanism involving HGF-mediated induction of the specific receptor and of the enzyme(s) involved in the proteolytic activation of pro-HGF.

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[2] G. Gaudino, L. Tessitore, C. Boccaccio, A. Follenzi, and P. M. Comoglio, manuscript in preparation.

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